Alterations in Hypertrophic Gene Expression by Dietary Copper Restriction in Mouse Heart (44492)

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Abstract. **Dietary copper (Cu) restriction causes a hypertrophic cardiomyopathy similar to that induced by work overload in rodent models. However, a possible change in the program of hypertrophic gene expression has not been studied in the Cu-deficient heart. This study was undertaken to fill that gap. Dams of mouse pups were fed a Cu-deficient diet (0.35 mg/kg diet) or a Cu-adequate control diet (6.10 mg/kg) on the fourth day after birth, and weanling mice continued on the dams′ diet until they were sacrificed. After 5 weeks of feeding, Cu concentrations were dramatically decreased in the heart and the liver of the mice fed the Cu-deficient diet. Corresponding to these changes, serum ceruloplasmin concentrations and hepatic Cu,Zn-superoxide dismutase activities were significantly ($P < 0.05$) depressed. The size of the Cu-deficient hearts was greatly enlarged as estimated from the absolute heart weight and the ratio of heart weight to body weight. The abundances of mRNAs for atrial natriuretic factor, β-myosin heavy chain, and α-skeletal actin in left ventricles were all significantly increased in the Cu- deficient hearts. Furthermore, Cu deficiency activated the expression of the c-*myc* oncogene in the left ventricle. This study thus demonstrated that a molecular program of alterations in embryonic genes, similar to that shown in the work-overloaded heart, was activated in the hypertrophied heart induced by Cu deficiency.** [P.S.E.B.M. 2000, Vol 223]

Dietary copper (Cu) restriction induces heart hypertrophy in animal models (1–3). This hypertrophy is predominantly concentric (1, 4, 5), where the ventricular and atrial walls are significantly thickened but lumen volumes are decreased. Moreover, increased mitochondrial volume density, increased ratio of mitochondria to myofibrils, and vacuolated mitochondria with disrupted cristae have been a common observation in the heart of Cu-deficient animals (4, 6, 7).

A concentric hypertrophy is a consequence of a variety of pathophysiological stimuli, such as myocardial infarction, hypertension, and aortic stenosis (8–10). Hypertrophy is an adaptive process in response to an increase in cardiac workload and elevated mechanical stress on cardiomyocytes. The increase in the mass and volume of individual myocytes results in an increase in heart weight without an increase in the number of cardiomyocytes. During the hypertrophic process, a distinct program of gene expression is activated. This eventually results in qualitative and quantitative alterations in contractile protein content, leading to a remodeling of the myocardium.

Altered gene expression in hypertrophied hearts induced by pressure overload has been studied extensively (11–14). A remarkable change is the re-expression of atrial natriuretic factor (ANF) in the ventricle of hypertrophied

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heart (11). ANF gene is expressed in both atrium and ventricle during embryonic development. Its expression is downregulated in the ventricle shortly after birth with the atrium as the primary site of ANF synthesis within the mature myocardium. After induction of ventricular hypertrophy, a re-expression of ANF occurs in the ventricular myocytes (14–16). Therefore, ANF gene expression by working ventricular cardiomyocytes has been considered mainly as a marker of myocardial hypertrophy and widely used as a prognostic parameter.

Work overload is accompanied by another change in gene expression, an induction of β-myosin heavy chain (β-MHC) (17–20). In particular, studies using the rat model showed that the initial low level of this isoform of myosin (0%–10%) in the ventricle makes the potential for its over-expression large (17). The accumulation of β-MHC reaches as much as 80% of total myosin in hypertrophied rat ventricles, and the amount of this accumulation is correlated with the degree of hypertrophy (17, 19, 20). Along with the changes in myosin gene expression, an induction of α-skeletal actin is another defined marker of ventricular hypertrophy by work overload. The abundance of α-skeletal actin mRNA is hardly detectable in the normal adult heart, but significantly increased at the onset of a pressure-overload ventricular hypertrophy in rats (21).

The triggers for changes in gene expression in the myocardium are critical elements for understanding the molecular mechanisms of myocardial remodeling. Because most studies have used pressure overload to initiate heart hypertrophy, searching for the triggers has focused on how a mechanical event alters the gene expression. In fact, the information currently available on molecular mechanisms of hypertrophy has been largely obtained from studies using healthy rats with aortic banding. However, the molecular and biochemical alterations that are triggered by artifactual hemodynamic modifications may not necessarily reflect pathophysiological triggers for the hypertrophic process.

The etiology of heart hypertrophy caused by Cu deficiency is quite different from that of pressure overload. Yet, the same concentric hypertrophy resulting from Cu deficiency and pressure overload demonstrates a common pathway that may be activated by both stimuli. It is interesting to note that the blood pressure is commonly lower in the Cu-deficient weanling rats, but higher in the pressure-overloaded animals. This indicates that high blood pressure may not necessarily be the cause of heart hypertrophy. As noted above, alterations in gene expression in cardiomyocytes play a critical role in the myocardial remodeling and cardiac hypertrophy. Therefore, it is important to know whether similar alterations in hypertrophic gene expression occur in the Cu-deficient heart. This information would indicate whether the heart hypertrophy induced by Cu deficiency shares similar molecular events with that of pressure overload. If similar molecular mechanisms of heart hypertrophy are involved in both Cu-deficient and pressure-overloaded animals, the Cu-deficient animal model would provide novel insights into the triggers for the myocardial remodeling. Therefore, in the present study, we examined alterations in hypertrophic gene expression along with analyses of cardiac morphological changes induced by dietary Cu restriction in the mouse model.

Materials and Methods

Animals and Treatment. FVB mice were bred and maintained at the University of Louisville animal facilities. Animals were housed in a plastic cage at 23°C on a 12:12-hr light:dark cycle. Dams of the pups were fed a Cu-adequate (CuA) or a Cu-deficient (CuD) diet starting on the fourth day postdelivery. The pups were weaned on the 21st day after birth, and the weanling mice were continued on the same diet until they were sacrificed at Week 3, 4, or 5 after CuD feeding (combined pre- and postweanling feeding). The animals had free access to doubly distilled water. Body weight gain of each mouse was monitored once a week during the experiment. The CuA and CuD diets (AIN-93 diet) were prepared according to Reeves *et al*. (22) and the primary ingredients were cornstarch (53%), casein (20%), sucrose (10%), and soybean oil (7%). Vitamins and minerals were provided in the diet. The CuA diet included an addition of 6 mg of Cu/kg diet, and the corresponding weight of cornstarch was added to the CuD diet. Analyses of the diets for Cu concentrations yielded 6.089 mg Cu/kg diet for CuA and 0.348 mg Cu/kg diet for the CuD diet. All procedures were approved by the AAALAC certified University of Louisville Institutional Animal Care and Use Committee.

Tissue Harvest. At the end of the feeding experiment and after an overnight fast, each animal was anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg body weight, Vet Labs, Lenexa, KS). Blood was withdrawn from the abdominal vena cava and serum was separated with a Serum Separator apparatus (Becton Dickenson, Inc., Rutherford, NJ) within 30 min. The inferior vena cava was cut open and the heart was perfused with cold 0.9% NaCl. The heart was then removed, opened, washed, dried with paper tissue, and weighed. Whole heart tissue was used for mineral and enzymatic determination, and left ventricle only was used for mRNA analysis. The liver was also perfused with cold 0.9% NaCl through the portal vein, and portions of liver were excised. All the tissue samples were either used immediately or placed in liquid nitrogen, then stored at 80°C for no longer than 36 hr before analysis.

Cu Concentrations. Cu concentrations in the heart and the liver were measured using inductively coupled argon plasma emission spectroscopy (model 35608, Thermo ARL-VG Elemental, Franklin, MA) after lyophilization and digestion of the tissues with nitric acid and hydrogen peroxide (23). Dietary Cu concentrations were analyzed by using a dry-ashing procedure (24), which was followed by dissolution of the residue in aqua regia and measurement by atomic absorption spectrophotometry (model 503, Perkin

Elmer, Norwalk, CT). Trace element contents of National Institute of Standards and Technology (NIST, Gaithersburg, MD) reference samples were within the specified ranges established by NIST, thus validating our assay procedure.

Serum Ceruloplasmin. Serum ceruloplasmin concentrations were determined by its p-phenylenediamine (PPD) oxidase activity (25). The oxidation of PPD at pH 5.4 yields a product that is readily detectable colorimetrically at 530 nm. The rate of product formation is proportional to the concentration of ceruloplasmin.

Superoxide Dismutase (Cu,Zn-SOD and Mn-SOD). Total SOD activity was determined by an NBT assay according to Spitz and Oberley (26). NaCN (5 m*M*) was used to assay Mn-SOD activity, and the Cu,Zn-SOD was calculated by subtracting the Mn-SOD activity from the total SOD activity. SOD activity was expressed as unit, as described previously (26).

Northern Blot Analysis. Total RNA was extracted from left ventricles of mice using an RNAzol B method. RNA was quantitated spectrophotometrically and confirmed by ethidium bromide staining of 18S and 28S ribosomal RNA. RNA was denatured in formaldehyde, fractionated by electrophoresis on 1.0% agarose gels, and transferred to nylon membranes. The oligonucleotides used as transcript-specific probes were as follows: ANF, 5′-AATGTGACCAAGCTGCGTGACACACCACAAGGGCTTAGGATCTTTTGCGATCTGCT-CAAG; β-MHC, 5′-GAAGCCCTCAGACCTGGAGCCTTTGCAACAGCCCTTTAGGTGGA-AGCAGAATAAAGC; and α-skeletal actin, 5′-TGGAGCAAAACAGAATGGCTGGCTTTAA-TGCTTCAAGTTTTCCATTTCCTTTCCACAGGG. All the probes were labeled with [γ-^{32}P]dATP using T_4 kinase (NEN, Boston, MA) and purified and precipitated by salt and ethyl alcohol. cDNA probes for c-*myc* and c-*fos* were obtained from the American Type Culture Collection (Rockville, MD), and labeled using [α-^{32}P]dCTP and Klenow enzyme in Random Primed Labeling Kit (Boehringer Mannheim, Germany). The probes were purified on Sephadex column by centrifugation at 1100*g* for 4 min. Hybridization and wash procedures were conducted using previously published methods (27). Autoradiographic images were scanned and analyzed using an MCID system from Imaging Research, Inc. (Ontario, Canada). The data were quantitated and compared between groups by a computerized densitometric analysis of the label intensity.

Data Analysis. A student *t* test was applied to all the experimental data analysis. Differences were considered significant at $P < 0.05$.

Results

Cu Concentrations in the Heart and the Liver of Mice Fed CuD Diet. To confirm the reduced Cu status induced by dietary Cu restriction, Cu concentrations in hearts and livers of mice fed the CuD diet for 3, 4, and 5 weeks (including dams and postweanling feeding) were compared with those from mice fed the CuA diet. As shown in Table I, both cardiac and hepatic Cu concentrations were significantly decreased in the mice fed the CuD diet for 3 weeks, and further lowered after the animals were fed the diet for 4 weeks. However, there was no further significant depression in animals fed the diet for 5 weeks (compared with that of 4 weeks). Neither cardiac nor hepatic Cu concentrations in the mice fed the CuA diet changed significantly during the feeding period.

Serum Ceruloplasmin Concentrations and Hepatic SOD Activities. To further determine Cu status and the biological consequences of Cu deficiency, the concentrations of Cu-dependent serum ceruloplasmin and activities of hepatic SOD were measured in mice fed the CuD diet for 5 weeks and compared with those in mice fed the CuA diet for the same time period. The serum ceruloplasmin concentrations were significantly ($P < 0.05$) decreased, and the hepatic Cu,Zn-SOD activities were also markedly ($P < 0.05$) depressed in the CuD-fed mice, compared with those in mice fed the CuA diet (Table II). However, the Mn-SOD activities were not altered in the liver of Cu-deficient mice (Table II).

The Development of Heart Hypertrophy by Dietary Cu Deficiency. Heart hypertrophy was determined by the absolute heart weight and the ratio of heart weight to body weight (Table I). At Week 3 postfeeding, both the heart weight and the ratio of heart weight to body weight were not significantly different between the mice fed the CuD and those fed the CuA diet. At Week 4, the ratios were

Table I. Effects of Cu Deficiency on Copper Concentrations in the Heart and Liver, Body Weight Gain and Heart Weight in Mice

	Cu deficiency			Cu adequacy		
	Wk 3	Wk 4	Wk 5	Wk 3	Wk 4	Wk 5
Heart Cu (μg/g)	15.7 ± 0.8*	9.7 ± 1.3*	7.1 ± 3.3*	21.8 ± 2.1	23.8 ± 0.6	25.1 ± 2.7
Liver Cu (μg/g)	10.5 ± 2.5*	5.6 ± 0.4*	5.6 ± 1.3*	39.2 ± 8.2	38.7 ± 3.1	37.8 ± 1.4
Body weight (g)	11.9 ± 2.1	15.1 ± 3.9	19.6 ± 2.5*	11.3 ± 1.6	19.1 ± 1.4	24.2 ± 3.5
BW gain (g)	ND	6.1 ± 0.9	7.6 ± 1.4*	ND	7.5 ± 1.9	9.8 ± 2.0
Heart weight (mg)	75 ± 11	122 ± 11	186 ± 25*	76 ± 10	111 ± 9	126 ± 15
HW/BW (mg/g)	6.3 ± 0.7	8.1 ± 0.6*	9.5 ± 1.4*	6.7 ± 0.3	5.8 ± 0.3	5.2 ± 0.3

Note. Tissue Cu concentrations are μg/g dry wt of tissues. *Significantly different from the CuA mice ($P < 0.05$).
Values are means ± SD ($n = 6$ for tissue Cu concentrations and $n = 10$ to 16 for others).
ND = not determined.

Table II. Changes in Hepatic Cu, Zn-SOD and Mn-SOD, and Serum Ceruloplasmin in Mice Fed CuD Diet for 5 Weeks

	Cu deficiency	Cu adequacy
Cu, Zn-SOD (U/mg protein)	88.5 ± 12.6*	150.9 ± 8.7
Mn-SOD (U/mg protein)	4.4 ± 1.1	4.9 ± 1.6
Ceruloplasmin (μg/ml)	23.7 ± 9.6*	109.3 ± 14.3

*Significantly different from the CuA mice ($P < 0.05$).
Values are means ± SD ($n = 12$).

significantly ($P < 0.05$) higher in CuD mice than in CuA mice, although the absolute heart weight of CuD mice was not statistically heavier than that of CuA mice. The difference in the ratio of heart to body weight between CuD and CuA mice was further exaggerated after they were fed the diet for 5 weeks, due to both a net increase in the heart weight ($P < 0.05$) and a decrease in the body weight again ($P < 0.05$) in the CuD mice. The CuD mouse hearts were obviously hypertrophic.

Alterations in Hypertrophic Gene Expression in the CuD Mouse Hearts. The abundances of mRNAs for ANF, β-MHC, and α-skeletal actin in left ventricles were examined by Northern blot analysis. Left ventricles were obtained from mice that were fed either CuD or CuA diet for 5 weeks, at which time these animals were 38 days old and the Cu-deficient hearts were hypertrophic. As shown in Figure 1 and the tabulated analysis, the abundance of mRNA for β-MHC was barely detectable in the left ventricles of mice fed the CuA diet, but dramatically elevated ($P < 0.05$) in the mice fed the CuD diet. Although the abundance of mRNA for α-skeletal actin in the left ventricle was detectable in both CuD and CuA mice, its expression was significantly ($P < 0.05$) enhanced in CuD mouse hearts. The abundance of mRNA for ANF in the left ventricle was also significantly ($P < 0.05$) elevated in the CuD mouse hearts.

Early Gene Expression. It has been recognized that some early genes, such as c-*myc* and c-*fos*, are upregulated in response to hypertrophic stimuli. Therefore, the abundances of mRNAs for c-*myc* and c-*fos* were determined in the left ventricles of mice fed either the CuD or CuA diet. The results shown in Figure 1 demonstrate that Cu deficiency indeed activated the expression of the c-*myc* gene. However, mRNA for c-*fos* was not detectable in either CuD or CuA mouse hearts (data not shown).

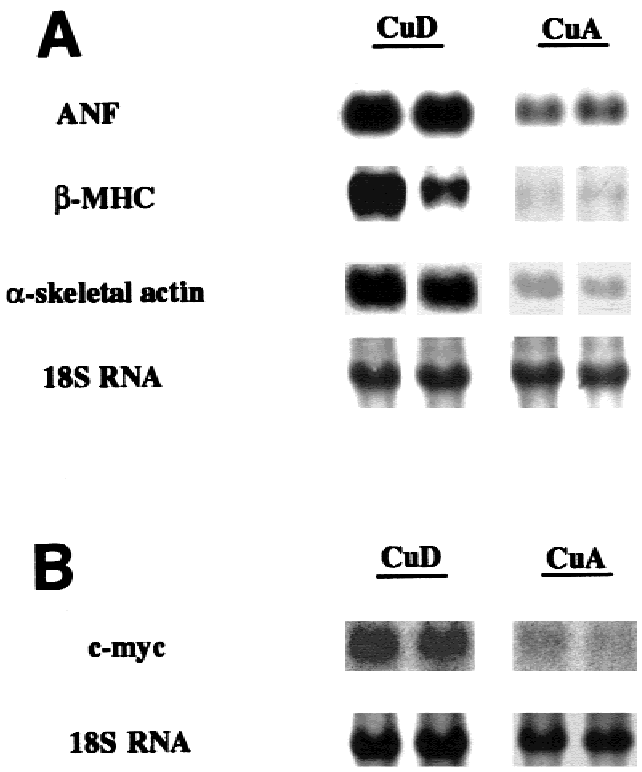

Figure 1. Cu deficiency–induced changes in embryonic gene expression in the left ventricle. Mice were fed either Cu-deficient (CuD) or Cu-adequate (CuA) diet for 5 weeks. The left ventricle was then removed from the sacrificed mice. (A) The abundances of mRNAs for ANF, β-MHC, and α-skeletal actin were detected by Northern blot analysis. Each transcript was measured five times with two to three animals at each time, and consistent results were obtained. (B) The abundances of mRNA for c-*myc* were detected by Northern blot analysis and repeated three times with two to three animals at each time. The representative autoradiography presents the results obtained from one experiment with two animals for each group. The tabulated data were obtained from all experiments by an autoradiographic image analysis system. The arbitrarily quantitative data (label intensity analyzed by computer) were compared between CuD and CuA groups and expressed as means ± SD, as follows:

	CuD	CuA	CuD/CuA
ANF	96.4 ± 0.6*	27.1 ± 2.3	3.56
β-MHC	90.6 ± 19.6*	6.8 ± 2.0	13.32
α-SA	105.5 ± 3.8*	35.2 ± 4.5	3.00
C-*myc*	10.4 ± 0.6*	3.0 ± 0.6	3.45

Note. Values are arbitrary densitometry units and expressed as means ± SD (n = 10–15 for the measurement of ANF, β-MHC and α-SA, and n = 6–9 for C-*myc*).
α-SA: α-skeletal actin.
*Significantly different from the CuA mice ($P < 0.05$).

Discussion

Heart hypertrophy induced by dietary Cu restriction is comparable to that induced by pressure overload in many respects, including morphological, biochemical, and physiological alterations (1). In the present study, heart hypertrophy induced by dietary Cu restriction was examined by following the time course of its progression. Mice fed a CuD diet for 5 weeks developed heart hypertrophy. However, significantly decreased Cu concentrations in the heart and the liver were detected on the third week after the mice were fed the CuD diet. The depletion of Cu in the tissues paralleled the decreased concentration of Cu-dependent serum ceruloplasmin and the suppressed activities of hepatic Cu,Zn-SOD. These parameters indicated that systemic Cu deficiency developed earlier than the development of heart hypertrophy.

The central features of the myocardial hypertrophic response to pressure overload are an increase in contractile protein content, the induction of contractile protein iso-

forms, and the re-expression of embryonic markers (11). In the case of Cu deficiency, the blood pressure of weanling animals is always lower than normal. It is therefore interesting to know whether the same program of gene expression occurs in the Cu-deficient hypertrophic heart as in the pressure-overloaded heart.

Upregulation of contractile protein genes results in accumulation of these proteins in myocardial cells. Corresponding to this accumulation, the abundances of mRNAs for these proteins also increase. In the present study, an increased expression of α-skeletal actin and β-MHC was observed in the left ventricle of the CuD mice. Actin is encoded by a multigene family in mammals. Two sarcomeric actins exist, the α-skeletal actin and the α-cardiac isoform. During the development of cardiac muscle in rodents, mRNA for α-skeletal actin accumulates in fetal and neonatal hearts, and α-cardiac actin mRNA becomes the predominant type in adult hearts (13). Therefore, the abundance of α-skeletal actin mRNA is hardly detectable in the normal adult rodent hearts. In the hypertrophied heart induced by pressure overload, this transcript is significantly elevated in the heart (13). In the present study, α-skeletal actin mRNA was detectable in the mice fed the CuA diet for 5 weeks. However, the abundance of this transcript was significantly elevated in the CuD mice. This result suggests that the switch from α-skeletal actin mRNA to α-cardiac actin mRNA as the predominant type in control (CuA) animals might have not completed and Cu deficiency caused a sustained expression of the α-skeletal actin.

In contrast to the α-skeletal actin mRNA, the abundance of β-MHC mRNA was barely detectable in the left ventricle of the mice fed the CuA diet for 5 weeks and was markedly elevated in the mice fed the CuD diet. It has been shown that in rodents, β-MHC is normally expressed in embryonic development. Reactivation of this contractile protein is a typical marker for heart hypertrophy induced by pressure overload in rat and rabbit models (13, 15, 17–19). In the CuD mice, this embryonic gene was apparently reactivated in the hypertrophied heart.

ANF is considered to be a noncontractile, embryonic marker that is upregulated during ventricular cell hypertrophy (11). The presence of ANF-mRNA and immunoreactive ANF in ventricular tissues has been demonstrated in pressure-overloaded, hypertrophied hearts. In fact, ventricular working cardiomyocytes represent a major source of circulating ANF during congestive heart failure. The abundance of ANF-mRNA in the CuD left ventricle was significantly elevated. This elevation, together with the increased abundances of mRNAs for β-MHC and α-skeletal actin, further demonstrated the similarity in the program of myocardial remodeling between pressure overload and CuD hypertrophied hearts.

Experimental hypertrophy using aortic banding has shown the expression of proto-oncogenes c-*fos*, c-*myc*, and c-*jun*, followed by the expression of fetal forms of α-actin and β-MHC protein (28, 29). In the present study, the abundances of mRNAs for c-*myc* and c-*fos* were analyzed. The expression of c-*myc* transcript was significantly activated in the CuD hypertrophied heart, but c-*fos* was not detectable. Although the link between early gene expression and the program of embryonic markers was not established in the present study, CuD hypertrophied hearts indeed displayed a pattern of myocardial remodeling programs similar to that of pressure overload.

Previous studies have suggested that increased mitochondrial volume is the primary cause of cardiac hypertrophy induced by Cu deficiency (4, 6, 7). Changes in mitochondrial metabolism and structure have also been observed in hypertrophied hearts induced by pressure overload. However, it has been demonstrated that the accumulation of the products overexpressed by embryonic genes is largely responsible for the myocardial hypertrophy (30). Thus, an interesting finding of this study is that the hypertrophic genes that are activated in pressure overloaded hearts were also significantly activated in CuD hearts. Therefore, although the contribution of mitochondrial changes to heart hypertrophy induced by Cu deficiency is an important element, the activation of embryonic genes must also be taken into account for the myocardial remodeling.

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